Effects of Ag Nanoparticle Flow Rates on the Progress of the Cell Cycle Under Continuously Flowing “Dynamic” Exposure Conditions

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In this study, we have investigated the flow rate effects of Ag nanoparticle (NP) suspensions on the progress of the cell cycle by using a microfluidic image cytometry (μIC)-based approach. Compared with the conventional “static” exposure conditions, enhancements in G2 phase arrest were observed for the cells under continuously flowing “dynamic” exposure conditions. The “dynamic” exposure conditions, which mimic in vivo systems, induced an enhanced cytotoxicity by accelerating G2 phase arrest and subsequent apoptosis processes. Moreover, we have also shown that the increases in delivered NP dose due to the continuous supply of Ag NPs contributed dominantly to the enhanced cytotoxicity observed under the “dynamic” exposure conditions, while the shear stress caused by these slowly flowing fluids (i.e., flow rates of 6 and 30 μL/h) had only a minor influence on the observed enhancement in cytotoxicity.

Key Words: Nanoparticles cytotoxicity, Image cytometry, Ag nanoparticle, Dynamic exposure conditions

Introduction

Recent advancements in nanoscience and nanotechnology have led to a large increase in the number of consumer products containing manufactured nanomaterials (MNs). However, despite an increased exposure to these “nano” products, our understanding of the absorption, distribution, metabolism, and excretion (ADME) of these nanomaterials in the human body are still limited. In particular, the toxicokinetics of nanoparticles (NP) are not yet fully understood. For instance, Ag NPs are one of the most commonly used NPs in numerous consumer and health care products because of their antimicrobial properties. However, many recent publications have presented strong evidence that Ag NPs are toxic to various mammalian cell lines via the formation of reactive oxygen species (ROS) and cell cycle arrest in the G2/M phases.1,2 In addition to the mass-based dose effects of Ag NPs, their physicochemical properties such as their hydrodynamic sizes, aggregation/agglomeration, and dissolution rates also play important roles in their observed toxicity.3,4 Recently, we reported that the physicochemical properties of Ag NPs, such as their degree of agglomerations, hydrodynamic sizes, and dissolution rates in cell culture media, also have strong influences on their cytotoxicity.5 However, our understanding of the detailed toxicity mechanisms and the roles of these physicochemical properties is still incomplete. In addition, most of these toxicity studies were performed under in vitro systems and the current knowledge on the toxicity mechanisms of Ag NPs under in vivo physiological conditions are limited.5,6 There is an urgent need to perform more toxicity evaluations of Ag NPs under in vivo systems or at least in vitro systems that can mimic in vivo conditions.

Among the many candidates for in vitro platforms that can simulate relevant in vivo conditions, microfluidics are considered to be one of the most promising techniques that can provide a deeper understanding of the interactions between mammalian cells and NPs under in vivo conditions, and their implications on resultant toxicities.6 Among the many dissimilarities between in vivo and in vitro systems, one of the key differences is the nature of the local cellular environment in which the assays are performed. Despite the many advantages of in vitro systems (e.g., they lend themselves to easier, cheaper, and more reproducible experiments with fewer ethical issues), their local cellular environments are too simple and static. In contrast, the local cellular environments of in vivo systems are much more complex with dynamic interactions between body fluids and various biological molecules. Under the continuously flowing “dynamic” exposure conditions of in vitro systems, two important differences in the local cellular environments are expected. First of all, due to the continuous supply of body fluids with constant levels of NPs, much higher doses of NPs are expected to be delivered, resulting in their greater accumulation. NPs in biological fluids generally aggregate/agglomerate to bigger particles and gravitationally settle on top of the adherent cells, which increases the delivered dose of NPs.7 Under the “static” exposure conditions of in vitro systems, this passive accumulation process combined with the active cellular uptake of NPs consume the NPs in the cell culture media. As a result, the total amount of delivered NP dose will reach its saturation level. In contrast, the continuous supply of fresh body fluids with constant level of NPs under the “dynamic” “in vivo” exposure conditions results in continuous increments of the effectively delivered NP dose to the adherent cells and may cause enhanced accumulation and/or toxicity of NPs. Secondly, the continuously flowing body fluids of in vivo systems may also cause significant...
shearing force on the adherent cells, which may also affect cytotoxicity. The shear stress on endothelial cells of the human vascular system is approximately ~1 N/m² and it is linearly related to the flow rates of body fluids. Kim et al. recently reported the shear stress-dependent cytotoxicity of mesoporous silica nanoparticles in endothelial cells under various flow conditions. The shear stress ranged from 0.5 to 6.6 N/m², which roughly corresponds to the exposure conditions in arterioles or capillaries. In microfluidic channels, it is possible to simulate a wide range of flow rates mimicking various regions of the human body. Higher flow rates are desirable to mimic vascular systems (e.g. arterioles or capillaries) with more shear stress on cells, while lower flow rate conditions are more appropriate to reflect slow-flowing body fluids across the organs and tissues of the body. Under slow-flowing conditions, agglomeration and sedimentation processes may have stronger effects on the actual delivered dose of Ag NPs, and the effects of shear stress caused by the flowing body fluids are negligible.

In this study, to investigate the effects of flow rates on the interactions between Ag NPs and mammalian cells, we have employed a microfluidic image cytometry (μFIC)-based cell cycle analysis approach. Particularly, building upon the recent study by Park and Yoon with emphasis on the more general application of MTT-based microfluidic image cytometry (μFIC) for the nanotoxicity study, we have focused on the effects of Ag NP flow rates on the progress of the cell cycle under continuously flowing “dynamic” exposure conditions that might reflect slowly flowing body fluids across the organs and tissues of the body.

Experimental

Design and Fabrication of the μFD. As shown in Figure S1(a), a simple microfluidic channel was used to investigate the interactions between HeLa cells and Ag NPs under continuous flow conditions. All of the experimental procedures including cell culture, exposure to Ag NPs, cell cycle analysis assay, and fluorescence image acquisition were performed in this simple microfluidic chip. The width, length, and height of the channel were 1000 μm, 2 x 10⁵ μm, and 165 μm, respectively, which corresponded to a total volume of 3.3 μL. The microfluidic device (μFD) was fabricated from polydimethylsiloxane (PDMS) using soft lithography technology, which was chemically bonded on the top of a glass coverslip (24 mm x 60 mm) substrate. SU-8 2150 photoresist (Microchem, Newton, MA, USA) spin-coated and photolithographically patterned on a silicon substrate was used as a cast for the top layer. After patterning, the master was silanized by exposure to tridecafluoro-1,1,2,2-tetrahydrooctyl (trichlorosilane) (Sigma Aldrich, St. Louis, MO, USA) and was positioned into the Petri dish (90 mm x 15 mm). Sylgard 184 PDMS prepolymer and curing agent (Dow corning, Midland, MI, USA) were thoroughly mixed in a 10:1 weight ratio. These mixtures were poured onto the cast, which was positioned on the Petri dish. They were then degassed in a vacuum chamber for 30 min before being baked at 60 °C. After curing, the PDMS layer was cut by a razor blade, peeled off from the master, and trimmed to size. Holes were punched out of the PDMS using flat-tip needles to form fluidic connection ports. The PDMS layer and the glass coverslip (24 mm x 60 mm) were treated with oxygen plasma (100 W, 0.2-1 mbar, 75 s; CUTE, Femto Science, South Korea) and finally bonded to each other.

Characterization of the Ag NPs. The Ag NPs used in this study (SARPU-200KW, lot no. SL-112B4DD01, ABC Nanotech, Korea) were purchased from ABC Nanotech. These Ag NPs were suggested as reference MNs by the Working Party on Manufactured Nanomaterials (WPMN) of the Organization for Economic Cooperation and Development (OECD) for international collaboration on the risk assessment of MNs. For the removal of agglomerated/aggregated particles from the as-received Ag NPs stock solution, the solution was filtered through a 0.2 μm syringe filter and stored at 4 °C in a dark environment. The total silver concentration was measured in an acidified solution [4% (v/v) HNO₃, 65% Ultrapure, Merck Chemicals, Germany] manufactured by ICP-AES (Optima-4300 DV; Perkin Elmer, Waltham, MA, USA). The shape and size distributions of the Ag NPs were measured using transmission electron microscopy (TEM; Hitachi H-7600, Tokyo, Japan). The UV-vis absorbances of the Ag NPs were measured using a UV-vis spectrophotometer (Mecasys Optizen-2120UV, Daejeon, Korea). The UV-vis absorption spectra and DLS measurements were taken at 25 °C in cuvettes with a 1 cm light path.

Cell Culture and Exposure to Ag NPs in the μFD. The μFD was initialized by filling media to remove dead volume and bubbles, and then placed inside a humidified incubator (Forma Scientific, Waltham, MA, USA) at 37 °C with 5% CO₂ for more than 6 h prior to use. The HeLa cell line (ATCC CCL-2) was purchased from KBR (Korea Biological Resource Center) and was cultured in fresh DMEM (Dulbecco’s Modified Eagle Medium) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% Penicillin-Streptomycin (Gibco, Grand Island, NY, USA). Cell suspensions were prepared at a concentration of 10⁴-10⁵ cells/ml and were introduced into the loading port of the μFD. The seeded μFD was placed into a humidified incubator at 37 °C with 5% CO₂ for 12 h until the next media exchange, which was performed two times per day. This cell culture cycle was repeated several times until the appropriate number of cells was obtained within the μFD. Due to the gas permeability of the PDMS, oxygen diffusion for the cell culture was sufficient. During the Ag NPs exposure periods, the μFD was also placed inside a CO₂ incubator with controlled humidity and temperature.

MTT Cell Viability Assay. The cytotoxicity of the Ag NPs under static exposure conditions were measured using a conventional MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-di-phenyltetrazolium bromide] assay protocol, which is based on the ability of mitochondrial dehydrogenase in viable cells to reduce MTT, a soluble yellow tetrazolium salt, into purple formazan crystals. HeLa cells were exposed to varying concentrations of Ag NPs for 24 h. Then, 100 μL of media were
Effects of Ag NP Flow Rates on the Progress of the Cell Cycle

removed from each well and 100 μL MTT [3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl-tetrazolium bromide, Sigma Aldrich, St. Louis, MO, USA] solution in cell culture media was added to each well. After 4 h of incubation at 37 °C (5% CO2), supernatant solutions were removed and 200 μL of DMSO (Sigma Aldrich, St. Louis, MO, USA) was added to dissolve the precipitated formazan crystals. Within 15 min, absorbance at the wavelength of 595 nm was measured by a microplate reader (Multiskan Ex, Thermo Scientific, Waltham, MA, USA).

Image Cytometry of Cell Cycle Analysis using the μFD. Vybrant DyeCycle™ Orange (Invitrogen, Eugene, OR, USA) was used as a fluorescent probe, which permeates the cell membrane and selectively binds to DNA. After exposure to Ag NPs, HeLa cells were washed twice with D-PBS solution and stained with 2 μL Vybrant DyeCycle™ Orange (10 μM diluted in phenol-red DMEM with FBS), which was then incubated for 30 min at 37 °C with 5% CO2. Fluorescence and optical images (×100) were acquired for all channel regions of the μFD using an inverted-type fluorescence microscope (IX51, Olympus, Japan) equipped with a cooled CCD camera (Retiga-2000RV 12-bit Mono Fast 1394 Cooled, Qimaging Ltd., Canada). The acquired fluorescent images were further analyzed to obtain the number of cells as well as information on individual cells (e.g. their integrated density parameters) by using image analysis software (CellProfiler 2.0, Broad Institute, Cambridge, MA, USA). In Figure S1(b), the Cell Profiler pipeline that automatically performed the image analysis is presented. This pipeline is composed of four subunits: load image, identify object, measure object properties, and export data. The acquired images were first loaded onto the Cell Profiler program and the cell nuclei in these images were identified via the Otsu global threshold method. Then, the various properties of each object (e.g. nucleus area, integrated density, mean intensity, and form factor) were measured and exported. Among these parameters, the integrated density (i.e. IntDen, cellular DNA content) was used for further classification of the cells into different phases of the cell cycle.

Results and Discussion

Physicochemical Characteristics and MTT Cell Viability of Ag NPs. Prior to investigating the effects of flow rate on the cytotoxicity of Ag NPs, we characterized their physicochemical properties. The core size distribution of the Ag NPs used in this study was found to be 8.3 ± 1.5 nm, and the particles were found to have a spherical shape. The characteristic UV-vis spectrum of the Ag NPs was also observed with an absorption maximum at a wavelength of 400 nm; in addition, the good dispersion stability of the Ag NPs in DMEM cell culture media was also confirmed in a previous study.

The MTT cell viability assays performed for the Ag NPs are presented in Figure 1(a), which show a typical dose-dependent decrease in cell viability. As shown in Figure 1(b)-(d), cell rounding and reduced cell populations were observed for the cells exposed to higher doses of Ag NPs (10 and 20 μg/mL), while in the control cells, cytokid-shaped cells clustered near each other and adhered well to surfaces. Based on these observations on cell viability and morphology, we chose 5 μg/mL Ag NPs as an appropriate dose level for the following μFIC-based cell cycle analysis under various flow rate conditions.

Effects of Ag NPs Under Static Exposure Conditions on the Progress of the Cell Cycle. To measure the cellular DNA contents, we used Vybrant DyeCycle™ Orange, which easily permeates cell membranes and specifically binds to DNA molecules in the nucleus. HeLa cells were exposed to various dose levels of Ag NPs for 24 h, with their optical and fluorescence images presented in Figure 2. In these optical images, the rounding of adherent cells were observed in a dose-dependent manner, which was followed by the condensation and crumpling of cells at higher Ag NP doses (15 and 20 μg/mL). In the fluorescence images under the same dose conditions, distinct changes in the amount of cellular DNA contents were observed. The nuclei became larger at lower doses of Ag NPs (5 and 10 μg/mL) and condensed into approximately half their original size at higher dose conditions (15 and 20 μg/mL). These observations in cell morphology and cellular DNA contents agree well with the MTT cell viability curve shown in Figure 1(a), where a rapid decrease in cell viability was observed between the dose levels of 5 and 15 μg/mL.

The histograms of cellular DNA contents shown in the bottom row of Figure 2 are results of image cytometric analysis of the fluorescence images shown in the second row of Figure 2. In these histograms, three distinct peaks that correspond to sub-G1 (cells with DNA contents less than 2N, frequently corresponds to the apoptotically fragmented cells), G1 (cells with DNA contents of 2N, the first phase within interphase, before the beginning of DNA synthesis), and G2 (cells with DNA contents of 4N, the phase between DNA synthesis and mitosis) were observed. The contributions of each cell cycle phase were extracted from these histograms and plotted in Figure 3. Similar to the trends shown in the histograms of Figure 2, an approximately ~20% decrease in the G1 phase with slight (~10%) increases in the G2 and sub-G1 phases were observed between the dose
levels of 5 and 10 µg/mL. However, during higher dosing conditions ([Ag NP] = 12.5-22.5 µg/mL), a rapid drop of the G2 phase down to approximately zero and an abrupt increase in the sub-G1 phase up to ~50% of the total cell population were observed, while only a slight decrease in the G1 phase was observed. Overall, under the “static” Ag NP exposure conditions, a cell cycle arrest in the G2 phase without apoptosis was observed at lower dose levels of 5-10 µg/mL, while apoptotic cell death accompanied by the transformation of G2 phase cells into the sub-G1 phase occurred at higher dose levels of 12.5-22.5 µg/mL.

Effects of Ag NP Flow Rates on the Progress of the Cell Cycle. To investigate the flow rate effects of Ag NP suspensions on the progress of the cell cycle (i.e. the changes in the cellular DNA contents), we performed μFIC-based cell cycle analyses of HeLa cells exposed to 5 µg/mL Ag NPs under flow conditions of 0, 6, and 30 µL/h. Particularly, in addition to the “static” exposure conditions in 96 well-plate (shown in Figure 2 and 3), we also performed control experiments under the “static exposure conditions in µFD with the same experimental setup with other dynamic exposure conditions” (shown in Figure 4, flow condition of 0 µL/h). HeLa cells under each flow condition were stained with Vybrant DyeCycle™ Orange and their representative fluorescence images are displayed in Figure 4(a)-(c).

As previously mentioned, Ag NPs under static exposure conditions resulted in a cell cycle arrest in the G2 phase even at low concentrations of Ag NPs (5-10 µg/mL), while apoptosis at higher doses of Ag NPs (12.5-22.5 µg/mL) were confirmed by the transformation of the G2 phase into the sub-G1 phase. Since the adherent HeLa cells exposed under

Figure 2. Optical (top row) and fluorescence (middle row) images of HeLa cells exposed to (a) 0 µg/mL, (b) 5 µg/mL, (c) 10 µg/mL, (d) 15 µg/mL, and (e) 20 µg/mL Ag NPs for 24 h. Histograms of cellular DNA contents were obtained from image cytometry analysis of the fluorescence images and displayed in the bottom row. In the fluorescence images, red arrows indicate cells in the G2 phase (4N), while white arrows indicate cells in the sub-G1 phase.

Figure 3. The effects of Ag NPs on the cell cycle of HeLa cells. Plot of the Ag NP dose-dependent changes in G1 (dark red), G2 (orange), and sub-G1 (light green) phase cells stained with Vybrant orange dye.

Figure 4. The effects of different flow rates on the cell cycles. The fluorescence cells were stained with Vybrant orange. The cells were treated with 5 µg/mL Ag NPs with (a) 0 µL/h, (b) 6 µL/h, and (c) 30 µL/h flow rates. (d) The plots represent Ag NPs flow rate-dependent changes in the G2 phase. Red arrows indicate the cells in the G2 phase (4N).
“dynamic” exposure conditions are thought to encounter harsher environments compared with those under “static” exposure conditions, all of the experiments under continuously flowing “dynamic” exposure conditions were performed for low dose levels (5 μg/mL Ag NP) that were just under the threshold for cell death. These slowly flowing “dynamic” exposure conditions are also preferred because they result in the least amount of adherent cell loss, which allow for more accurate and reproducible image cytometric analysis of cell cycle changes.

Under these continuously flowing “dynamic” exposure conditions, there is a continuous supply of Ag NPs as well as shearing forces on the adherent cells induced by the flowing media fluids. Temporal changes in the % G2 phase under the flow rates of 0, 6, and 30 μL/h were processed and displayed in Figure 4(d). Under the “static” exposure condition (i.e., flow rate of 0 μL/h), slow and steady incremental increases in the % G2 phase was observed after 6 h (up to 37.11% at 12 h). For intermediate flow rates (6 μL/h), the incremental increase in the % G2 phase reached up to 41.11% after 9 h. Under the highest flow rate (30 μL/h), a much faster increase in the % G2 phase (a 12.94% increase from 28.24% to 41.18% during the first 3 h) was observed. Additionally, the initial increment rates seem to strongly dependent on the flow rates of Ag NP suspensions, which were found as 7.57, 14.17 and 40.86 for the flow rates of 0, 6 and 30 μL/h, respectively. Compared with the “static” exposure conditions, 1.87- and 5.40-fold increases in G2 phase arrest were observed for the continuously flowing “dynamic” exposure conditions. These observations indicate that the “dynamic” exposure conditions of in vivo systems may have a stronger cytotoxicity by accelerating G2 phase arrest and subsequent apoptosis.

The flow conditions used in this study were deliberately chosen based on the results of previous studies, so that the continuous supply of Ag NPs in this study contribute dominantly to the cytotoxicity with negligible contributions from shear stress. According to Walker et al., flow rates of 60-1200 μL/h in microfluidic channels correspond to shear stresses in the range of 0.0229-0.458 N/m², while the shear stress on endothelial cells in the human vascular system is approximately ~1 N/m². Previously, Kim et al. reported the shear stress-dependent cytotoxicity of mesoporous SiO₂ NPs under flow conditions that roughly correspond to those of arterioles and capillaries (0.5-6.6 N/m²). Compared to typical flow rates in blood vessels, the flow conditions used in this study (0-30 μL/h) are very slow and can be considered to reflect slowly flowing body fluids across the organs and tissues of the body, rather than fluids in the human vascular system.

As previously mentioned, under continuously flowing “dynamic” exposure conditions, two important differences in local cellular environments are expected. A continuous supply of fresh medium with constant levels of Ag NPs may cause much higher delivered doses and the subsequent accumulation of Ag NPs, while the flowing fluids may also induce significant shearing force on the adherent cells. In this study, we have designed “dynamic” exposure conditions with the continuous supply of Ag NPs that minimizes the contribution of shear stress. To confirm the minimal or negligible contribution of shear stress, control experiments under the highest flow rate (30 μL/h) without Ag NPs (0 μg/mL) were also conducted and compared with those with Ag NPs (5 μg/mL) in Figure 5. Under these exposure conditions, the control experiments are expected to be affected only by the shear stress of the flowing fluids, while those exposed to 5 μg/mL Ag NPs under the same flow rate are expected to be affected by both shear stress and the Ag NPs. As shown in Figure 5, only a 3.60% increase (from 30.99% to 33.59%) in the % G2 phase was observed for the control experiment, while a ~6-fold higher increase (14.48%, from 28.24% to 42.72%) in the % G2 phase was observed for the HeLa cells exposed to 5 μg/mL Ag NPs with a flow rate of 30 μL/h. These observations under the given experimental conditions (Ag NP concentration = 5 μg/mL exposure time < 5 h, flow rate = 30 μL/h) suggested that the shear stress under the given “dynamic” exposure conditions in this study had only a minimal influence, and confirmed that the higher delivered doses of Ag NPs due to the continuous supply of the suspension provided the dominant contribution on the enhanced cytotoxicity observed under the “dynamic” exposure conditions.

Conclusion

In this study, we have investigated the interactions between Ag NPs and mammalian cells by using a microfluidic image cytometry (μFIC)-based cell cycle analysis approach. Particularly, building upon our recent studies, we further focused on the effects of Ag NP flow rates on the progress of the cell cycle under continuously flowing “dynamic” exposure conditions that might reflect slowly flowing body fluids across the organs and tissues of the body. Compared with the
“static” exposure conditions, enhancements in G2 phase arrest were clearly observed for the continuously flowing “dynamic” exposure conditions, indicating that the “dynamic” exposure conditions under in vivo systems may have enhanced cytotoxicity by accelerating G2 phase arrest and subsequent apoptosis. We suggest that the enhancements in G2 phase arrest under the continuously flowing “dynamic” exposure conditions is mainly due to the continuous increments of the effectively delivered NP dose to the adherent cells, which may cause enhanced accumulation and/or toxicity of NPs.

**Supporting Information.** Supporting figure S1 is available free of charge.

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